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**UTILITY  
PATENT APPLICATION  
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	835-005.003
First Inventor	Martin E. Davis
Title	Enzymatic Treatment of Whey Protein for the Production of Antihypertensive Peptides and the Resulting Products
Express Mail Label No.	EL628639435US

**APPLICATION ELEMENTS**

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ Fee Transmittal Form (e.g., PTO/SB/17) (Submit an original and a duplicate for fee processing) NO FEE
  2. ☒ Applicant claims small entity status. ENCLOSURE See 37 CFR 1.27
  3. ☒ Specification [Total Pages 18]  
(preferred arrangement set forth below)
    - Descriptive title of the invention
    - Cross Reference to Related Applications
    - Statement Regarding Fed sponsored R & D
    - Reference to sequence listing, a table, or a computer program listing appendix
    - Background of the Invention
    - Brief Summary of the Invention
    - Brief Description of the Drawings (if filed)
    - Detailed Description
    - Claim(s)
    - Abstract of the Disclosure
  4. ☐ Drawing(s) (35 U.S.C. 113) [Total Sheets ☐
  5. Oath or Declaration [Total Pages ☐  - a. ☐ Newly executed (original or copy)
  - b. ☐ Copy from a prior application (37 CFR 1.63 (d)) (for continuation/divisional with Box 17 completed)
    - i. ☐ **DELETION OF INVENTOR(S)**  
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)
6. ☐ Application Data Sheet. See 37 CFR 1.76

**ADDRESS TO:**Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

7. ☐ CD-ROM or CD-R in duplicate, large table or Computer Program (Appendix)
8. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
  - a. ☐ Computer Readable Form (CRF)
  - b. Specification Sequence Listing on:
    - i. ☐ CD-ROM or CD-R (2 copies), or
    - ii. ☐ paper
  - c. ☐ Statements verifying identity of above copies

**ACCOMPANYING APPLICATION PARTS**

9. ☐ Assignment Papers (cover sheet & document(s))
10. ☐ 37 CFR 3.73(b) Statement (when there is an assignee) ☐ Power of Attorney
11. ☐ English Translation Document (if applicable)
12. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
13. ☐ Preliminary Amendment
14. ☒ Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
15. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
16. ☐ Other: .....

17. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP)

of prior application No. .... / .....

Prior application information

Examiner .....

Group / Art Unit .....

For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

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Signature

Date 30 October 2000

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# FEE TRANSMITTAL for FY 2001

Patent fees are subject to annual revision.

## Complete if Known

Application Number	
Filing Date	
First Named Inventor	Martin E. Davis
Examiner Name	
Group Art Unit	
Attorney Docket No.	835-005.003

TOTAL AMOUNT OF PAYMENT (\$).00

## METHOD OF PAYMENT

1. ☐ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

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- ☐ Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17  
☐ Applicant claims small entity status. See 37 CFR 1.27

2. ☒ Payment Enclosed ☒ NOT ENCLOSED

☐ Check ☐ Credit card ☐ Money Order ☐ Other

## FEE CALCULATION

### 1. BASIC FILING FEE

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
101 710	201 355	Utility filing fee	
106 320	206 160	Design filing fee	
107 490	207 245	Plant filing fee	
108 710	208 355	Reissue filing fee	
114 150	214 75	Provisional filing fee	

SUBTOTAL (1) (\$).00

### 2. EXTRA CLAIM FEES

Total Claims  Extra Claims  Fee from below  Fee Paid   
Independent Claims  - 3\*\* =  Fee Paid   
Multiple Dependent  Fee Paid

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description
103 18	203 9	Claims in excess of 20
102 80	202 40	Independent claims in excess of 3
104 270	204 135	Multiple dependent claim, if not paid
109 80	209 40	** Reissue independent claims over original patent
110 18	210 9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) (\$).00

\*or number previously paid, if greater; For Reissues, see above

## FEE CALCULATION (continued)

### 3. ADDITIONAL FEES

Large Entity Fee Code (\$)	Small Entity Fee Code (\$)	Fee Description	Fee Paid
105 130	205 65	Surcharge - late filing fee or oath	
127 50	227 25	Surcharge - late provisional filing fee or cover sheet	
139 130	139 130	Non-English specification	
147 2,520	147 2,520	For filing a request for <i>ex parte</i> reexamination	
112 920*	112 920*	Requesting publication of SIR prior to Examiner action	
113 1,840*	113 1,840*	Requesting publication of SIR after Examiner action	
115 110	215 55	Extension for reply within first month	
116 390	216 195	Extension for reply within second month	
117 890	217 445	Extension for reply within third month	
118 1,390	218 695	Extension for reply within fourth month	
128 1,890	228 945	Extension for reply within fifth month	
119 310	219 155	Notice of Appeal	
120 310	220 155	Filing a brief in support of an appeal	
121 270	221 135	Request for oral hearing	
138 1,510	138 1,510	Petition to institute a public use proceeding	
140 110	240 55	Petition to revive - unavoidable	
141 1,240	241 620	Petition to revive - unintentional	
142 1,240	242 620	Utility issue fee (or reissue)	
143 440	243 220	Design issue fee	
144 600	244 300	Plant issue fee	
122 130	122 130	Petitions to the Commissioner	
123 50	123 50	Petitions related to provisional applications	
126 240	126 240	Submission of Information Disclosure Stmt	
581 40	581 40	Recording each patent assignment per property (times number of properties)	
146 710	246 355	Filing a submission after final rejection (37 CFR § 1.129(a))	
149 710	249 355	For each additional invention to be examined (37 CFR § 1.129(b))	
179 710	279 355	Request for Continued Examination (RCE)	
169 900	169 900	Request for expedited examination of a design application	

Other fee (specify)

\* Reduced by Basic Filing Fee Paid SUBTOTAL (3) (\$).00

## SUBMITTED BY

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Signature		Date	30 October 2000		

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(835-005.003)

**UNITED STATES PATENT APPLICATION**

of

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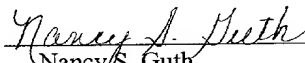
**ENZYMATIC TREATMENT OF WHEY PROTEINS  
FOR THE PRODUCTION OF ANTIHYPERTENSIVE  
PEPTIDES AND THE RESULTING PRODUCTS**

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October 30, 2000

  
Nancy S. Guth

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## DESCRIPTION

### ENZYMATIC TREATMENT OF WHEY PROTEINS FOR THE PRODUCTION OF ANTIHYPERTENSIVE PEPTIDES AND THE RESULTING PRODUCTS

#### Background of The Invention

The invention relates to a method for suppressing angiotensin-converting enzyme (ACE), a composition effective for this purpose and a method for preparing the composition, specifically by enzymatic conversion of whey proteins.

Hypertension has been reported to be the most important cause of human deaths in industrialized countries. (See, for example, Laragh, J. H., 1979. *L'hypertension. Recherche*, 105 (10): 1068-1076.) Nearly 30% of the fatalities among adults would result from hypertension or from its renal, coronary or neurological complications. The elucidation of the physiological mechanisms responsible for hypertension has lead the pharmaceutical industry to propose angiotensin converting enzyme (ACE) -inhibitory substances. ACE catalyses the degradation of angiotensin I into angiotensn II, a strong vasoconstrictor.

Peptides found in Brazilian snake venom have been identified as the most effective natural substance for the inhibition of ACE. (See, Ferreira, S. H., Bartelt, D. C., Greene, L. J., 1970. Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. *Biochemistry*, 9 (13): 2583-2593.) The inhibitory effect of natural peptides has been related to their binding at the active site of ACE. (See, Maubois, J. L., Léonil, J., Trouvé, R., Bouhallab, S., 1991. Les peptides du lait à activité physiologique III. Peptides du lait à effet cardiovasculaire: activités antithrombotique et antihypertensive. *Lait*, 71: 249-255.)

A structure-function study of these various bioactive peptides has suggested that they often possess a Pro-Pro, Ala-Pro or Ala-Hyp at their C-terminal sequence. (See, Maruyama, S., Suzuki, H., 1982. A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. *Agric. Biol. Chem.*, 46 (5): 1393-1394; and Oshima, G., Shimabukuro, H., Nagasawa, K. 1979. Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. *Biochim. Biophys. Acta*, 566: 128-137.) The occurrence of proline might also contribute to the ACE-inhibitory activity of peptides derived from food proteins. (See, Kohmura, M., Nio, N., Kubo, K., Minoshima, Y., Munekata, E., Ariyoshi, Y. 1989. Inhibition of angiotensin-converting enzyme by synthetic peptides of human  $\beta$ -casein. *Agric. Biol. Chem.*, 53 (8): 2107-2114.)

Maruyama and Suzuki [*supra*] have evidenced such amino acid sequences in peptides from tryptic casein hydrolysates. The authors have shown that the peptide f23-34 from  $\kappa_{s1}$  casein (bovine, variant B), possesses ACE-inhibitory activity estimated by an  $IC_{50}$  value (concentration needed to inhibit 50% ACE activity) of 77 $\mu$ M. Numerous other studies followed this work and revealed other ACE-inhibitory peptides in casein hydrolysates. In a recent review, Nakano has reported the occurrence of 18 distinct milk protein-derived peptide sequences, found in sour milk, and which have been shown to possess ACE-inhibitory activity. (Nakano, T., 1998, Milk derived peptides and hypertension reduction. *Int. Dairy J.*, 8: 375-381.)

However, only a few studies have reported the occurrence of ACE-inhibitory activities among whey proteins hydrolysates. Abubakar, *et al.*, have determined the ACE-inhibitory activity in whey protein hydrolysates using seven different enzymes: trypsin, proteinase-K, actinase-E, thermolysin, papain, pepsin and chymotrypsin. It was shown that the specificity of the enzyme had a pronounced effect on the resulting ACE-inhibitory activity of the hydrolysate, and that the biological activity was originating from the major whey proteins ( $\beta$ -lg,  $\alpha$ -la, BSA, Ig) and not from the caseinomacropeptide. (Abubakar, A., Saito, T., Aimar, M. V., Itoh, T. 1996. New derivation of the inhibitory activity against angiotensin converting enzyme (ACE) from sweet cheese whey. *Tohoku J. Agric. Res.*, 47 (1-2): 1-8.) More recent work from Abubakar, *et al.*, has allowed the identification of nine peptide sequences, namely  $\beta$ 2-microglobulin (f18-20),  $\beta$  -

lactoglobulin (f78-80), serum albumin (f221-222),  $\beta$ -casein (f59-61, f59-64, f62-63, f80-90, f157-158, f205-206), among which  $\beta$ -lactoglobulin (f78-80) showed the strongest antihypertensive activity in spontaneously hypertensive rats. (Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., Itoh, T., 1998, Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *J. Dairy Sci.*, 12: 3131-3138.) Finally, Mullaly *et al.*, have demonstrated that a peptidic fraction, isolated by using RP-HPLC, from a tryptic hydrolysate prepared with bovine  $\beta$ -lactoglobulin had an  $IC_{50}$  value of 159.8  $\mu$ mol/L, compared to Captopril, a commercial drug commonly used in hypertension treatment, which has an  $IC_{50}$  of 0,006  $\mu$ mol/L. (Mullally, M. M., Meisel, H., FitzGerald, R. J., 1997. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine  $\beta$ -lactoglobulin. *FEBS Letters*, 402: 99-101.) Mass spectrometry analyses have allowed the identification of peptide f142-148 from  $\beta$ -lactoglobulin as being responsible for the ACE-inhibitory activity in tryptic hydrolysates of  $\beta$ -lactoglobulin. The same peptidic sequence obtained by chemical synthesis showed an  $IC_{50}$  of 42.6  $\mu$ mol/L.

### Summary of the Invention

It is an object of the invention to provide improvements in the field of suppressing angiotensin-converting enzyme (ACE).

It is an object of the invention to provide a method for suppressing angiotensin-converting enzyme.

It is another object of the invention to provide a composition effective for suppressing angiotensin-converting enzyme.

It is yet another objective of the invention to provide a method for preparing a composition effective for suppressing angiotensin-converting enzyme, specifically by enzymatic conversion of whey proteins.

These and other objects are accomplished by the invention, by improvements which enable the production of an ACE-suppressing composition by the hydrolysis of whey protein hydrolysate, the recovery of the ACE-suppressing composition, and a regimen for use of the ACE-suppressing composition.

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The process for preparing the ACE-suppressing composition comprises: preparing an aqueous solution of whey protein isolate and a proteolytic enzyme; holding said solution under conditions effective to partially hydrolyze said whey protein isolate to provide a hydrolysate having increased ACE-suppressing activity in mammals; and recovering said hydrolysate from said solution. The proteolytic enzyme is inactivated as necessary, preferably by heating. The hydrolysate is preferably dried for use in a regimen which comprises oral administration to a mammal, such as a human or a domestic pet such as a dog or cat, in amounts and at intervals effective to suppress ACE-activity. Many of the preferred aspects of the invention are described below.

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### Detailed Description of The Invention

Whey protein isolates (WPI) can be obtained from commercial-scale fractionation of cheese whey by various processes, including ion-exchange processing using cationic and/or anionic resins selected for the intended functionality of the isolate. (Pearce, R.J., 1992, Whey protein recovery and whey protein fractionation, In *Whey and Lactose Processing*, JG Zadow, Ed., Elsevier, London, 271-316.) Commercial WPI products issued from ion-exchange processing, such as BiPRO™ (Davisco Foods International, LeSueur, MN), are characterized by a high protein content (>94% w/w), low ash content (<3%), traces (<1%) of fat and lactose. The protein distribution of a typical WPI shows 73%  $\beta$ -lactoglobulin, 15%  $\alpha$ -lactalbumin, and the remaining 12% is composed of bovine serum albumin, immunoglobulins and caseinomacropptide.

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BiPRO™ whey protein isolate is the preferred source of whey protein isolate for use in the invention and is available from Davisco Foods International, Inc., with offices at 11000 W. 78th Street, Suite 210, Eden Prairie, Minnesota 55344. The preferred BiPRO™ whey protein isolate has a (PDCAAS) Protein Digestibility Corrected Amino

Acid Score of 1.14. The fat and lactose levels are less than 1%. The *BiPRO*<sup>TM</sup> whey protein isolate is prepared by ion-exchange technology, and contains about 91% (w/w)  $\beta$ -lactoglobulin. Preferably, the whey protein isolate employed according to the invention will contain at least 70% and preferably at least 80%, *e.g.*, 85-95%,  $\beta$ -lactoglobulin, with the remaining comprising  $\alpha$ -lactalbumin, serum albumin, immunoglobulins and caseinomacropptide. *BiPRO*<sup>TM</sup> is essentially undenatured and is fully soluble over the pH range 2.0 to 9.0, and has the following analysis:

Analysis*	Specification	Typical Range	Test
<b>Method</b>			
Moisture (%)	5.0 max.	$4.7 \pm 0.2$	Vacuum Oven
Protein, dry basis (N x 6.38)(%)	95.0 min.	$97.5 \pm 1.0$	Combustion
Fat (%)	1.0 max.	$0.6 \pm 0.2$	Mojonnier
Ash (%)	3.0 max.	$1.7 \pm 0.3$	Gravimetric
Lactose (%)	1.0 max.	< 0.5	by difference
pH	6.7 - 7.5	$7.0 \pm 0.2$	10% Sol. @ 0°C.

\* All results reported "AS IS" basis except where noted.  
Standard Methods for the Examination of Dairy Products, 16th Edition.

As noted, whey protein isolates other than *BiPRO*<sup>TM</sup> can be employed and where used preferably have similar analyses to that above, varying by from 0 to 25%, *e.g.*, from 5 to 10%, or less, from the above Typical Range values. A suitable whey protein isolate can be produced having similar properties through a selective ion exchange process that selects the primary functional proteins - beta-lactoglobulin and alpha-lactalbumin - for concentration and spray drying. Such a process is described in U. S. Patent No. 4,154,675 to Jowett, *et al.*, and U. S. Patent No. 4,218,490 to Phillips, *et al.* If properly produced, whey protein fractions having lower protein contents, *e.g.*, as low as 35%, might be employed. In addition,  $\beta$ -lg produced by ion exchange separation can also be employed, but is less preferred than the *BiPRO*<sup>TM</sup> whey protein isolate.



On a more detailed analysis of *BiPRO*<sup>TM</sup> whey protein isolate, the following is found for each 100 grams of whey protein isolate:

**Component**

5	Calories	374
	Calories From Total Fat	3
	Total Fat (g)	0.3
	Saturated Fat (g)	0.2
	Cholesterol (mg)	10
10	Sodium (mg)	600
	Potassium (mg)	120
	Total Carbohydrates (g)	0
	Dietary Fiber (g)	0
	Sugars (g)	0
15	Protein (g)	93
	Vitamin A (IU)	20
	Vitamin C (mg)	2.0
	Calcium (mg)	120
	Iron (mg)	5
20	Phosphorus (mg)	25
	Magnesium (mg)	15
	Ash (g)	2
	Moisture (g)	5

25 And, to provide an amino acid profile of the preferred *BiPRO*<sup>TM</sup> whey protein isolate, samples were subjected to drying for 24 hours in a dessicator over phosphorous pentoxide and sodium hydroxide. The dry samples were hydrolyzed in HCl vapor (6N HCl with 1% phenol and 0.5% sodium sulfite) under Argon atmosphere. After 20 hours of hydrolysis at 110 degrees Celsius, the samples were dissolved in 200 u1 of Beckman  
30 Na-S sample buffer. This acid hydrolysis method destroys tryptophan.

Analyses were conducted on a Beckman 6300 Amino Acid Analyzer. Norleucine was used as an internal standard. The analysis showed the following:

	Grams Amino Acid Per		
	Amino Acid	100g protein	100g powder
5	Alanine	7.6	7.01
	Arginine	2.0	1.84
	Aspartate	10.1	9.31
10	Cysteine/Cystine	4.3	3.96
	Glutamine	14.3	13.18
	Histidine	1.6	1.48
	Isoleucine *	5.4	4.98
	Leucine *	13.7	12.63
15	Lysine *	9.6	8.85
	Methionine *	2.4	2.21
	Phenylalanine *	3.1	2.86
	Proline	4.5	4.14
	Serine	4.90	4.52
20	Threonine *	5.30	4.89
	Tyrosine	2.90	2.67
	Valine *	5.60	5.16
	<b>Totals</b>	<b>100.10</b>	<b>92.29</b>

25 \* Essential Amino Acids

Again, when whey protein isolates other than *BiPRO*<sup>™</sup> are employed, they preferably have similar analyses to that above, varying by from 0 to 25%, *e.g.*, from 5 – 10%, or less, from the above values.

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Enzymatic digests of *BiPRO*<sup>™</sup> and of commercial  $\beta$ -lg-rich product were prepared using animal, bacterial and fungal proteases, in order to determine the potential

of these commercial substrates for the preparation of peptide mixtures having antihypertensive activities. The objective of the work was to determine the ACE-inhibitory activity of various hydrolysates generated by enzymatic hydrolysis from whey protein isolates obtained by ion-exchange chromatography, in comparison with other commercially-available whey protein hydrolysates.

## MATERIALS AND METHODS

Whey protein hydrolysate WPH 917 (84.5% protein w/w) was obtained from New Zealand Milk Product Inc. (Santa Rosa, USA). Whey protein hydrolysate LE80GF (80.0% w/w) was obtained from DMV International (New-York, USA). Whey protein isolate (*BiPRO*<sup>TM</sup>) and  $\beta$ -lactoglobulin-rich product were obtained from Davisco Foods International (Le Sueur, MN, USA). Purified peptidic sequence *Ala-Leu-Pro-Met-His-Ile-Arg* modelling the peptide f-142-148 from  $\beta$ -lg was chemically-synthesized by the *Service de séquence de peptides de l'Est du Québec* (Ste-Foy, Qc, Canada). HEPES Sodium salt, Hippuryl-L-Histidyl-L-Leucine, and Angiotensin Converting Enzyme (from rabbit lung) were purchased from Sigma Chemical Co. (St. Louis, USA). All other products used were analytical grade.

Whey proteins (*BiPRO*<sup>TM</sup> or  $\beta$ -lg) were solubilized at 20% W/V, adjusted to pH 8.0 or 8.5 by using a mixture of NaOH and KOH 4N and maintained at temperatures between 40°C and 50°C corresponding to the optimal temperature of the enzymes used. Table 1 reports the characteristics of the enzymes used for the preparation of the enzymatic hydrolysates for the study. *BiPRO*<sup>TM</sup> and  $\beta$ -lg-rich product were utilized for the preparation of 601 and 605, but only *Bipro*<sup>TM</sup> was used for 603K. The protein solutions were incubated with the proteases at an enzyme:substrate ratio of 1:800 for AS-601, 1:50 for AS-603K and 1:100 for AS-605K. The enzymatic hydrolysis was performed under pH-stat conditions until a degree of hydrolysis (DH) of 5.5-6.5% for AS-601 and under a combination of pH-stat and osmometry methods until a DH of 11.0-12.5% for AS-603K, and a DH of 19.5-20.5% for AS-605K. The hydrolysis reaction was stopped at the selected DH values by means of heat treatment (75 to 85°C for 15 s) in a plate heat exchanger to inactivate the enzyme and followed by cooling and storage at 5-10

°C until further processing. The resulting hydrolysates were further spray dried and handled as powdered ingredient. Fractions can be taken based on molecular weight and tested for relative activity, with the most active fractions selected.

- 5 A typical analysis for the AS-601K and AS-603K products prepared from *BiPRO*<sup>TM</sup> whey protein hydrolysate are given below.

	Analysis* (AS-601K)	Specification	Typical Range	Test Method
10	Moisture (%)	5.0 max.	4.0 ± 0.5	Vacuum Oven
	Total Nitrogen (TN), %	14.1 min.		Combustion
	Protein dry basis (N x 6.38)(%)	90.0 min.	91.0 ± 0.5	Calculated
	Amino Nitrogen (AN), %	1.7 min.		Formol
15	Titration			
	AN/TN, %	12.0 min.	12.0 - 15.0	Calculated
	Degree of Hydrolysis, %	5.0 min.	5.5 - 6.5	OPA Method
	Fat (%)	1.0 max	< 1.0	Mojonnier
	Ash (%)	6.0 max.	5.5 ± 0.3	Standard **
20	Lactose (%)	1.0 max.	< 1.0	by difference
	pH	8.5 max.	8.0 ± 0.2	10% Sol. @ 20°C

25	<b>Molecular Weight Profile (HPLC) Range (Daltons)</b>	<b>Soluble Peptides***</b>
	> 5000	50 - 55%
	2000 – 5000	15 - 20%
	< 2000	30 - 35%

\* All results reported "AS IS" basis except where noted.

30 \*\* Standard Methods for the Examination of Dairy Products, 16th Edition.

\*\*\* Percent of total soluble peptides in 0.45 µ filtrate.

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<b>Analysis*(AS-603K)</b>	<b>Specification</b>	<b>Typical Range</b>	<b>Test Method</b>
Moisture (%)	5.0 max.	4.0 ± 0.5	Vacuum Oven
Total Nitrogen (TN), %	14.1 min.		Combustion
Protein, dry basis (N x 6.38) %	94.0 min.	95.0 ± 0.5	Calculated
Amino Nitrogen (AN), %	2.2 min.	2.4 ± 0.2	Formol Titration
AN/TN, %	15.8 min.	16.3 ± 0.5	Calculated
Degree of Hydrolysis, %	7.7 min.	8.7 ± 1.0	OPA Method
Fat (%)	1.0 max.	<1.0	Mojonnier
Ash (%)	5.0 max.	3.5 ± 0.3	Gravimetric
Lactose (%)	1.0 max.	<1.0	by difference
pH	7.5 max.	7.0 ± 0.2	10% Sol. @ 20°C.
Scorched Particles	15 mg/25 g max.	7.5 mg	ADPI

#### **Molecular Weight Profile (HPLC)**

<b>Range (Daltons)</b>	<b>Peptides</b>
>10,000	30 – 35%
5000 – 10,000	10 – 15%
2000 – 5000	25 – 30%
<2000	25 – 30%

**Table 1 Characteristics of the enzyme sources used for the preparation of hydrolysates**

Enzyme (Name, Supplier)	Source	Optimal pH	Temp. (°C)	Hydrolysate
Trypsin VI Trypsin Activity 2,400 U/mg minimum Chymotrypsin activity 350 U/mg minimum <i>Canadian Innovatech Inc</i> <i>Abbotsford, BC, Canada</i>	Porcine	8.0	37	AS-601 ( <i>BiPRO</i> <sup>TM</sup> )  AS-601 ( $\beta$ -lg)
Protease P Amano-6 Proteinase activity 60,000 units/g minimum <i>Amano, Enzyme Co. Ltd</i> <i>Rochester, IL, USA</i>	Fungal	7.5	45	AS-605K ( <i>BiPRO</i> <sup>TM</sup> )  AS-605K ( $\beta$ -lg)
Multifect Activity 3,000 GSU/ml minimum (Note: GSU is Genencor Subtlisin Units --- developed internally by Genencor) <i>Genencor International</i> <i>Rochester, NY, USA</i>	Bacterial	8.5	50	AS-603K ( <i>BiPRO</i> <sup>TM</sup> )

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#### **Determination of ACE-inhibitory activity**

The ACE-inhibitory activity was measured *in vitro* by a spectrophotometric assay according to the method of Cushman and Cheung. (Cushman, D.N., Cheung, H.S. 1971 Spectrophotometric assay and properties of the angiotensin converting enzyme of rabbit lung. *Biochemical Pharmacology*, 20: 1637-1648.) According to this method, hippuric acid is liberated from hippuryl-L-histidyl-L-leucine (HHL) by the enzymatic reaction of ACE. After extraction by ethyl acetate and removal of ethyl acetate by heat evaporation, hippuric acid is dissolved in deionized water.

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Table 2 summarizes the experimental conditions used for the assay. Absorbance of the hippuric acid solution at 228 nm was determined by spectrophotometry.

**Table 2      Assay conditions for the measurement of ACE-inhibitory activity**

Reagent volumes (μl)	Sample	Control	Blank
Substrate solution <sup>1</sup>	200	200	200
Sample solution <sup>2</sup>	50	—	—
HEPES-HCl Buffer <sup>3</sup>	—	50	50
Deionized water	20	20	20
mixed using vortex and equilibrated to 37°C			
Stopping solution <sup>4</sup>	—	—	300
ACE solution <sup>5</sup>	30	30	30
mixed using vortex and incubated at 37°C for 20 min			
Stopping solution	300	300	—
Total volume (μl)	600	600	600

1: HHL was dissolved in HEPES-HCl Buffer to obtain a final concentration of 0.3% (w/v).

2: Samples were diluted at the appropriate concentration with HEPES-HCl Buffer.

3: HEPES Sodium Salt (50 mM) with 300 mM NaCl, pH adjusted at 8.3 with 1M HCl

4: 1M HCl.

5: ACE from rabbit lung dissolved in HEPES-HCl at a final concentration of 0.33 unit ml<sup>-1</sup>.

Inhibitory activity was calculated according to the following equation:

$$\text{Inhibitory activity (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / (A_{\text{control}} - A_{\text{blank}})] \times 100 \quad [1]$$

where A represents absorbance. A plot of the inhibitory activity (%) *versus* log<sub>10</sub> of sample concentration (mg powder ml<sup>-1</sup>) was generated using 6 different concentrations of samples for BiPRO™, AS-601 (BiPRO™β-lg), commercial hydrolysates (WPH 917, LE80GF) and of synthetic peptide (f142-148) from β-lg. Each concentration was tested in

triplicate and the mean value was plotted in the curves. The IC<sub>50</sub> value (expressed in terms of mg powder ml<sup>-1</sup>, defined as the concentration of inhibitor which gives 50% inhibition of ACE activity, was calculated using the linear regression equations of the curves.

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The linear regression equations corresponding to all the hydrolysates under study are reported in Table 3. The data show that non-hydrolyzed *BiPRO*<sup>TM</sup> has very low ACE-inhibitory activity (IC<sub>50</sub> 380 mg powder. ml<sup>-1</sup> ) in comparison with all other products under study. The highest ACE-inhibitory activity was obtained with synthetic peptide f142-148 of  $\beta$ -lg for which 50% of inhibition was already obtained at the lowest concentration (IC<sub>50</sub> 0.04 mg powder.ml<sup>-1</sup>). The ACE-inhibitory effectiveness of the hydrolysates under study followed the order : AS-603K (*BiPRO*<sup>TM</sup>)> AS-605K (*BiPRO*<sup>TM</sup>) >AS-601 (*BiPRO*<sup>TM</sup>) > AS-605K ( $\beta$ -lg ) > AS-601 ( $\beta$ -lg ) > LE80GF > WPH 917.

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Table 3, as follows presents the linear regression equations ( $y = m \ln X + b$ ) of the ACE-inhibitory activity curves obtained with synthetic peptide  $\beta$ -lg (f142-148), in comparison with the hydrolysates under study.

Table 3

Sample	Slope <i>m</i>	Y-intercept <i>b</i>	Reg. Coeff. <i>R</i> <sup>2</sup>
$\beta$ -lg f142-148	10.35	84.4	.83
AS-601 ( <i>BiPRO</i> <sup>TM</sup> )	15.39	62.4	.99
AS-605 ( <i>BiPRO</i> <sup>TM</sup> )	14.76	63.0	.99
AS-603K ( <i>BiPRO</i> <sup>TM</sup> )	14.33	67.9	.96
AS-601 ( $\beta$ -lg)	17.69	51.9	.98
AS-605 ( $\beta$ -lg)	16.98	60.7	.98
WPH 917 (NZMP)	19.40	44.8	.96
LE80GF (DMV)	18.01	50.7	.97

The values of IC<sub>50</sub> calculated for all the samples under study are listed in Table 4.

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The samples of *BiPRO*<sup>TM</sup> showed lower ACE-inhibitory activity with an IC<sub>50</sub> of 376 mg



powder.ml<sup>-1</sup>), whereas synthetic peptide f142-148 of  $\beta$ -lg showed the lowest value (0.04 mg powder.ml<sup>-1</sup>). The hydrolysates derived from *BiPRO*<sup>TM</sup> or  $\beta$ -lg-rich products all gave lower IC<sub>50</sub> values (0.29 to 0.90 mg powder.ml<sup>-1</sup>) than the other commercial hydrolysates (0.96 and 1.30 mg powder.ml<sup>-1</sup>). Also the hydrolysates derived from *BiPRO*<sup>TM</sup> led to lower IC<sub>50</sub> values than those derived from the  $\beta$ -lg-rich product (ex. AS-601 *BiPRO*<sup>TM</sup>=0.45 vs AS-601 $\beta$ -lg= 0.90 mg powder.ml<sup>-1</sup>).

**Table 4** Values of IC<sub>50</sub> for various whey protein products

Sample	Description	IC <sub>50</sub> (mg powder ml <sup>-1</sup> )
<i>BiPRO</i> <sup>TM</sup>	whey protein isolate	376.7
AS-601 ( <i>BiPRO</i> <sup>TM</sup> )	hydrolyzed <i>BiPRO</i> <sup>TM</sup>	0.45
AS-601 ( $\beta$ -lg)	hydrolyzed $\beta$ -lg	0.90
AS-605 ( <i>BiPRO</i> <sup>TM</sup> )	hydrolyzed <i>BiPRO</i> <sup>TM</sup> , Kosher certified	0.42
AS-605 ( $\beta$ -lg)	hydrolyzed $\beta$ -lg, Kosher certified	0.53
AS-603K ( <i>BiPRO</i> <sup>TM</sup> )	Hydrolyzed <i>BiPRO</i> <sup>TM</sup> , Kosher certified	0.29
WPH 917 (NZMP)	whey protein hydrolysate	1.30
LE80GF (DMV)	whey protein hydrolysate	0.96
$\beta$ -lg f142-148	synthetic peptide	0.04

## DISCUSSION

Our observations show that the enzymatic hydrolysis of whey proteins issued from ion-exchange chromatography, such as *BiPRO*<sup>TM</sup> whey protein isolate generates hydrolysates having superior ACE-inhibitory activities, compared to commercial hydrolysates prepared with other sources of whey proteins. Moreover, it appears that *BiPRO*<sup>TM</sup> whey protein isolate is a better substrate than  $\beta$ -lg-rich product for the preparation of hydrolysates with antihypertensive activity, as seen by the lower IC<sub>50</sub> values obtained (Table 4) for AS-601, AS-603K and 605K prepared from *Bipro*<sup>TM</sup> whey protein isolate. This observation was unexpected since it was hypothesized that a  $\beta$ -lg-rich product would offer a higher potential for producing ACE-Inhibiting peptides, especially the fragment  $\beta$ -lg 142-148 liberated by trypsin (Mullally, M. M., Meisel, H.,

FitzGerald, R. J., 1997. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine  $\beta$ -lactoglobulin. *FEBS Letters*, 402: 99-101). In fact, the hydrolysates having the lowest  $IC_{50}$  (0.29 mg powder  $ml^{-1}$ ) were obtained by hydrolysis with bacterial protease (AS-603K) and not with trypsin (AS-601). Altogether, our results suggest the occurrence of a synergistic effect when *BiPRO*<sup>TM</sup> whey protein isolate is used as the substrate, but the explanation for this phenomenon is not clear.

*BiPRO*<sup>TM</sup> whey protein isolate and others similarly prepared are preferred for a composition with regard to principal protein composition ( $\beta$ -lg,  $\alpha$ -la, etc.), and content of minor proteins (lactoferrin, lactoperoxidase, immunoglobulins) or peptidic fragments (caseinomacropeptides, proteoses peptones, etc.) which may be precursors of the production of peptides with very strong ACE-inhibition activity during enzymatic hydrolysis. Some of these minor proteins may be at a lower concentration in the  $\beta$ -lg-rich product, as a result of the different fractionation conditions.

Also, it must be emphasized that the enzymes used for the preparation of enzymatic hydrolysates from *BiPRO*<sup>TM</sup> whey protein isolate (601, 605K and 603K) respectively Trypsin VI, P Amano 6 and Multifect have very different specificities. Trypsin is known to cleave only the peptidic bonds in the vicinity of Arg and Lys, whereas the two other enzymes have a much broader specificity and will lead to a greater number of shorter peptides. The recent work from Abubakar, *et al.*, *supra*, suggests that short molecules such as tri- and even di-peptides can have an antihypertensive effect. It is clear that the fragments 142-148 and 78-80 of  $\beta$ -lg are not the only ACE-inhibitors responsible for the antihypertensive properties of all whey protein hydrolysates.

The synergistic effect on ACE-inhibitory potential of whey protein hydrolysates when *BiPRO*<sup>TM</sup> whey protein isolate is used may also be originating from its low mineral content, especially with regards to divalent cations such as calcium (15-20 meq/kg) or magnesium (<1 meq/kg). These physicochemical conditions may prevent the occurrence of *peptide-peptide* interactions and therefore preserve the high ACE-inhibitory potential of the hydrolysate. This hypothesis will be further investigated by comparing the mineral

composition of *BiPRO*<sup>TM</sup> whey protein isolate with that of  $\beta$ -lg-rich product which showed a lower ACE-inhibitory potential.

This study has shown that the enzymatic hydrolysis of *BiPRO*<sup>TM</sup> leads to a synergistic effect in terms of ACE-inhibitory potential of the hydrolysates obtained. It appears that the synergy could result from unique compositional characteristics brought by the ion-exchange process in relation with the presence of minor proteins or fragments.

The above description is intended to enable the person skilled in the art to practice the invention. It is not intended to detail all of the possible modifications and variations which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such modifications and variations be included within the scope of the invention which is seen in the above description and otherwise defined by the following claims. The claims are meant to cover the indicated elements and steps in any arrangement or sequence which is effective to meet the objectives intended for the invention, unless the context specifically indicates the contrary.

**CLAIMS**

1. A process for preparing an ACE-suppressing composition comprising:  
preparing an aqueous solution of whey protein isolate and a proteolytic enzyme;  
holding said solution under conditions effective to partially hydrolyze said whey protein isolate to provide a hydrolysate having increased ACE-suppressing activity in mammals;  
and  
recovering said hydrolysate from said solution.
2. A process according to claim 1 wherein the proteolytic enzyme is inactivated.
3. A process according to claim 1 wherein the proteolytic enzyme is inactivated by heating following hydrolysis.
4. A process according to claim 1 wherein the hydrolysate is dried.
5. A whey protein hydrolysate as prepared according to claim 1.
6. A treatment for a mammal to suppress ACE, said regimen comprising:  
orally administering to the mammal, a product prepared according to claim 1 in amounts and at intervals effective to suppress ACE-activity.

**ABSTRACT OF THE DISCLOSURE****5                    ENZYMATIC TREATMENT OF WHEY PROTEINS  
                     FOR THE PRODUCTION OF ANTIHYPERTENSIVE  
                     PEPTIDES AND THE RESULTING PRODUCTS**

Enzymatic digests of whey protein concentrates were prepared using animal, bacterial and fungal proteases, and evaluated for antihypertensive activities. The highest  
10    ACE-inhibitory activity was obtained with the purified peptide  $\beta$ -lg (f142-148) obtained  
by chemical synthesis, for which an  $IC_{50}$  value of 0.04 mg powder.ml<sup>-1</sup> was found. The  
hydrolysates derived from *BiPRO*<sup>TM</sup> whey protein isolate and  $\beta$ -lg both gave higher  
antihypertensive activities ( $IC_{50}$  values of 0.29 to 0.90 mg powder.ml<sup>-1</sup>) than the other  
hydrolysates tested ( $IC_{50}$  values of 0.96 and 1.30 mg powder.ml<sup>-1</sup>). The recovered  
15    hydrolysate can be used to treat hypertension in mammals such as humans and domestic  
pets such as dogs and cats.